ELSEVIER

Food Chemistry 69 (2000) 33–36

Food Chemistry

www.elsevier.com/locate/foodchem

# Anthocyanin trisaccharides in blue berries of *Vaccinium padifolium*

Luis Cabrita, Nils Åge Frøystein, Øyvind M. Andersen*

*Department of Chemistry, University of Bergen, Allégt. 41, 5007 Bergen, Norway*

Received 19 April 1999; received in revised form 16 September 1999; accepted 16 September 1999

**Abstract**

Delphinidin 3-*O*-α-rhamnoside, malvidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) and the 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of cyanidin, petunidin and peonidin were isolated by various chromatographic techniques from the edible berries of *Vaccinium padifolium*. Their complete structures were elucidated mainly by one- and two-dimensional nuclear magnetic resonance spectroscopy. Together they account for 7% of the anthocyanin content in this species. No anthocyanidin 3-triglycoside, 3-rutinoside or 3-rhamnoside had previously been found in the genus *Vaccinium*. The 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of petunidin and peonidin are novel compounds. 

## 1. Introduction

*Vaccinium padifolium* is a deciduous shrub endemic to Madeira island (Portugal). Its berries have been used as food preserves and in local ethnopharmacology (cough, colds, bronchitis, dysentery) (Rivera & Obón, 1995), and exported for commercial production of ophthalmic specialities (Vieira, 1992). We have recently reported twenty different anthocyanins, including the 3-*O*-β-glucopyranosides, 3-*O*-β-galactopyranosides, 3-*O*-β-arabinopyranosides and 3-*O*-sambubiosides (2″-*O*-β-xylopyranosyl-*O*-β-glucopyranosides) of delphinidin, cyanidin, petunidin, peonidin and malvidin to occur in its berries (Cabrita & Andersen, in press).

The aim of this study is to present the isolation and structure elucidation of five additional anthocyanins in *V. padifolium*, which have not been reported in this genus before, including two novel anthocyanidin 3-triglycosides.

## 2. Materials and methods

### 2.1. Extraction and separation

Ripened berries of Uveira, (*Vaccinium padifolium*) (Ericaceae) were collected in Madeira island (Portugal) in October 1995 and stored at −20°C. The berries (100 g) were extracted ten times with 100 ml MeOH containing 1% TFA at 4°C. The combined extract was filtered, concentrated under reduced pressure and partitioned against ethyl acetate. The purified extract was then adsorbed on a column packed with Amberlite XAD-7, washed with water, eluted with MeOH containing 0.1% TFA and dried. The sample was fractionated on a Sephadex LH-20 column (1000×50 mm) using step elution with 20–60% MeOH–$H_2O$ (containing 0.1% TFA) and the collected fractions were further purified by semi-preparative HPLC.

### 2.2. HPLC and TLC

Analytical and semi-preparative high performance liquid chromatography (HPLC) separations were performed on a HP-1050 module system (Hewlett–Packard) using an ODS Hypersil column (200×4.6 mm, 5 μm) and diode array detection. Two solvents were used for elution: A. $HCO_2H$:$H_2O$ (1:9, v/v); B. MeOH:$HCO_2H$:$H_2O$ (5:1:4, v/v). Analytical HPLC: the elution profile was 0–4 min, 10% B in A (isocratic); 4–21 min, 10–100% B in A (linear gradient), 21–25 min, 100% B (isocratic). The flow rate was 1.2 ml $min^{-1}$. Semi-preparative HPLC: 0–4 min, 10% B in A (isocratic); 4–30 min, 10–100% B, 30–35 min, 100% B. Prior to injection all samples were filtered through a 0.45 μm Millipore membrane filter. Peak percentages were estimated from several HPLC chromatograms obtained using gradients

* Corresponding author.

0308-8146/00/$ - see front matter 
PII: S0308-8146(99)00230-7

with different steepness, since no single elution program could resolve all peaks.

Thin-layer chromatography (TLC) was carried out on microcrystalline cellulose F (Merck) with the solvents 1-BuOH:HOAc:$H_2O$ (4:1:5, upper phase) and $HCO_2H$:-conc. HCl:$H_2O$ (1:1:2).

### 2.3. Spectroscopy

UV-vis absorption spectra (240–600 nm, 2 nm steps) were recorded in 0.01% conc. HCl in MeOH. On-line absorbance signals were recorded for every second nm between 500 and 540 nm.

The NMR experiments (DQF-COSY, 1D TOCSY, $^1$H-$^{13}$C HSQC, $^1$H-$^{13}$C HMBC, $^{13}$C SEFT) were obtained at 600.13 and 150.92 MHz for $^1$H and $^{13}$C, respectively, on a Bruker DRX-600 instrument at 25°C. The deuteriomethyl $^{13}$C signal and the residual $^1$H signal of the solvent ($CF_3CO_2D$:$CD_3OD$; 1:19, v/v) were used as secondary references (49.0 and 3.4 ppm from TMS, respectively).

Table 1
UV-visible data and retention times (HPLC) for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), petunidin (**2**) and peonidin (**4**), delphinidin 3-rhamnoside (**3**) and malvidin 3-rutinoside (**5**)

| Anthocyanin | Vis max (nm) | $A_{440}/A_{max}$ (%) | HPLC, $t_R$ (min) |
|---|---|---|---|
| **1** | 531 | 27 | 18.2 |
| **2** | 540 | 30 | 19.3 |
| **3** | 540 | 20 | 20.1 |
| **4** | 531 | 31 | 24.8 |
| **5** | 540 | 22 | 26.8 |

## 3. Results and discussion

The berries were extracted with an acidified methanolic solution. The extract was purified by liquid–liquid partition and washed on XAD-7. The eluate was concentrated under vacuum to dryness (1.2 g), and separation of the sample (1.0 g) on Sephadex LH-20 yielded 44 fractions that were analysed by TLC and analytical HPLC. After further purification by semi-preparative reversed-phase HPLC, fractions 4–7 yielded pigments **1** (1.1 mg), **2** (3.3 mg) and **4** (2.5 mg); fractions 6–9 afforded

Table 2
$^1$H NMR chemical shifts and $^1$H-$^1$H coupling constants for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), peonidin (**4**) and petunidin (**2**), malvidin 3-rutinoside (**5**) and delphinidin 3-rhamnoside (**3**)

| | **1** δ (ppm) *J* (Hz) | **4** δ (ppm) *J* (Hz) | **2** δ (ppm) *J* (Hz) | **5** δ (ppm) *J* (Hz) | **3** δ (ppm) *J* (Hz) |
|---|---|---|---|---|---|
| *Aglycone* | | | | | |
| 4 | 8.97 s | 9.04 s | 8.99 s | 9.10 s | 9.04 s |
| 6 | 6.76 d 1.8 | 6.77 d 1.8 | 6.76 d 1.8 | 6.82 d 1.8 | 6.74 *d* 2.0 |
| 8 | 6.98 dd 2.0, 0.7 | 7.03 d 0.9 | 7.00 d 1.1 | 78.09 d 1.5 | 6.94 dd 1.8, 0.6 |
| 2′ | 8.13 d 2.2 | 8.14 d 2.2 | 7.86 d 2.2 | 8.13 s | 7.65 s |
| 5′ | 7.1 d 8.8 | 7.14 d 8.8 | | | |
| 6′ | 8.37 dd 8.8, 2.2 | 8.49 dd 8.7,2.2 | 7.99 d 2.2 | 8.13 s | 7.65 s |
| OMe | | 4.12 s | 4.12 s | 4.10 s | |
| *3*-O-*glucoside* | | | | | |
| 1″ | 5.53 d 7.7 | 5.53 d 7.7 | 5.54 d 7.7 | 5.41 d 7.7 | |
| 2″ | 4.06 dd 7.7, 9.2 | 4.02 dd 7.7,91.2 | 4.03 dd 7.7, 9.2 | 3.73 dd 7.7,9.2 | |
| 3″ | 9.86 t 9.2 | 3.86 t 9.2 | 3.85 t 9.2 | 3.63 t 9.2 | |
| 4″ | 3.56 t 9.2 | 3.54 t 9.2 | 3.55 t 9.4 | 3.48 t 9.2 | |
| 5″ | 3.82 ddd 9.2, 5.5, 1.8 | 3.82 ddd 8.8, 6.6, 1.8 | 3.82 ddd 9.2, 6.6, 1.8 | 3.81 ddd 9.5, 6.6, 1.8 | |
| 6″ A | 4.13 dd 11.4, 1.8 | 4.13 dd 11.4, 1.8 | 4.12 dd 11.4, 1.8 | 4.14 dd 11.4, 1.8 | |
| 6″ B | 3.70 dd 11.4, 5.5 | 3.69 dd 11.4, 6.6 | 3.70 dd 11.4, 6.6 | 3.68 dd 11.4, 6.6 | |
| *2″*-O-*xylosyl* | | | | | |
| 1‴ | 4.85 d 7.7 | 4.85 d 7.7 | 4.77 d 7.7 | | |
| 2‴ | 3.25 dd 7.7, 9.2 | 3.23 dd 7.7, 9.0 | 3.23 dd 7.7, 9.2 | | |
| 3‴ | 3.40 t 9.2 | 3.39 t 9.2 | 3.36 t 9.2 | | |
| 4‴ | 3.49 ddd 10.3, 9.2, 5.5 | 3.48 ddd 10.2, 9.2, 5.5 | 3.42 ddd 10.3, 9.2, 5.5 | | |
| 5‴ A | 3.78 dd 11.4, 5.5 | 3.77 dd 11.4, 5.5 | 3.67 dd 12.1, 5.5 | | |
| 5‴ B | 3.14 dd 11.4, 10.3 | 3.14 dd 11.4, 10.3 | 3.05 dd 12.1, 10.3 | | |
| *6″*-O-*rhamnosyl* | | | | | 3-*O*-rhamnoside |
| 1′ᵛ | 4.73 d 1.5 | 4.73 d 1.5 | 4.73 d 1.5 | 4.73 d 1.5 | 5.82 d 1.5 |
| 2′ᵛ | 3.86 dd 1.5, 3.3 | 3.85 dd 1.5, 3.3 | 3.85 dd 1.5, 3.3 | 3.87 dd 1.5, 3.3 | 4.35 dd 1.5, 3.3 |
| 3′ᵛ | 3.71 dd 3.3, 9.5 | 6.39 dd 3.3, 9.2 | 3.69 dd 3.3, 9.5 | 3.69 dd 3.3, 9.2 | 4.02 dd 3.3, 9.3 |
| 4′ᵛ | 3.41 t 9.3 | 3.40 t 9.5 | 3.40 t 9.4 | 3.40 t 9.3 | 3.66 t 9.4 |
| 5′ᵛ | 3.65 dd 9.2, 6.2 | 3.69 dd 9.5, 6.2 | 3.64 dd 9.2, 6.2 | 3.31 dd 9.5, 6.2 | 3.73 dd 9.5, 6.2 |
| 6′ᵛ | 1.23 d 6.2 | 1.23 d 6.2 | 1.22 d 6.2 | 1.23 d 6.2 | 1.37 d 6.2 |

pigment **5** (4.9 mg), and pigment **3** (7.5 mg) was recovered from fractions 35–37 (Table 1).

The low-field part of the $^1$H NMR spectrum of **4** showed 6 resonances (see Table 2). On the basis of chemical shifts and coupling-patterns, the signals of the AMX system at 8.14, 7.14 and 8.49 ppm were assigned to H-2′, H-5′ and H-6′, respectively, the 2H AX system at 7.03 and 6.77 ppm to H-8 and H-6, and the singlet at 9.04 ppm to H-4. Together with the 3H singlet at 4.12 ppm (OMe), which in the HMBC spectrum correlated with C-3′ (4.1/149.8 ppm) (Fig. 1), these assignments confirmed the identity of the aglycone to be peonidin.

The anomeric proton signals in the $^1$H NMR spectrum of **4** appear considerably downfield for the other sugar resonances, and thus the three doublets at 5.53, 4.86 and 4.73 ppm, together with the integration data, defined a 1:1:1:1 ratio between the peonidin aglycone and the three sugars. Starting from the doublet at 4.73 ppm (H-1$^{IV}$) the observed crosspeak with the signal at 3.85 ppm in the DQF-COSY permitted assignment of H-2. The chain of coupled protons H-2, H-3, H-4, H-5 and H-6, was, thereafter, assigned using the same spectrum (Table 2). Subsequently the chemical shifts of the corresponding carbon atoms (Table 3) were assigned from the HSQC experiment, which together with $^1$H-$^1$H coupling constants were in agreement with an α-linked rhamnopyranosyl. On the basis of a combination of COSY, TOCSY, HSQC, HMBC and SEFT NMR spectra it was possible to assign all the $^1$H (Table 2) and $^{13}$C (Table 3) resonances of the other sugar moieties, which were determined to be β-glucopyranosyl and β-xylopyranosyl units. The HMBC spectrum of **4** revealed cross-peaks establishing that the glucosyl was attached to the aglycone 3-position H-1″/C-3, 5.6/145.4 ppm), while the xylosyl and rhamnosyl residues were attached to the 2″- (H-1‴/C-2″, 4.9/81.4 ppm) and the 6″-positions (H-1$^{IV}$/C-6″, 4.8/67.7 ppm) of the glucosyl moiety, respectively (Fig. 1). Thus, the identity of **4** was found to be peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranoside).

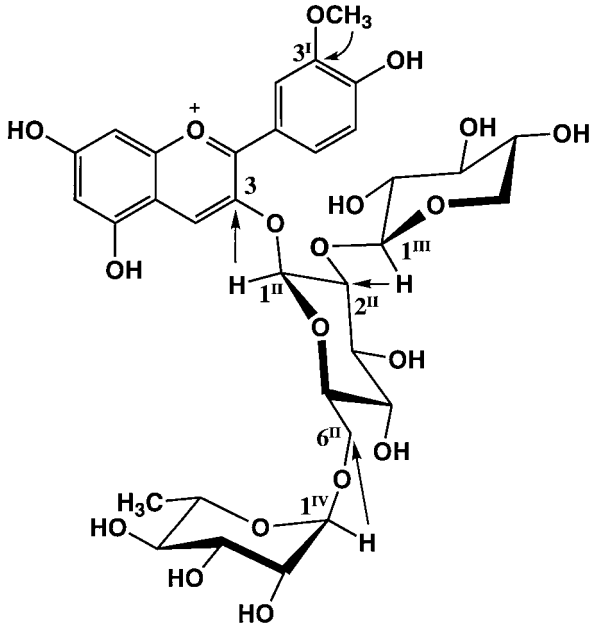


Fig. 1. The arrows refer to proton and carbon cross-peaks in the HMBC NMR spectrum of peonidin 3-*O*-(6″-α-rhamnosyl-2″-β-xylosyl-β-glucoside) showing linkage points between the different moieties.

The $^1$H and $^{13}$C shifts of **2** (Tables 2 and 3) were assigned by the same one- and two-dimensional homo- and heteronuclear NMR techniques as used for **4**, showing the same sugar moiety. The aglycone B-ring signals of **2** at 7.86 and 7.99 ppm were assigned to H-2′ and H-6′, respectively, and the cross-peak between the

Table 3
$^{13}$CNMR chemical shifts for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), peonidin (**4**) and petunidin (**2**), malvidin 3-rutinoside (**5**) and delphinidin 3-rhamnoside (**3**)

| | **1** δ (ppm) | **4** δ (ppm) | **2** δ (ppm) | **5** δ (ppm) | **3** δ (ppm) |
|---|---|---|---|---|---|
| *Aglycone* | | | | | |
| 2 | 163.64 | 164.29 | 164.38 | 163.98 | 164.49 |
| 3 | 145.31 | 145.32 | 145.34 | 145.74 | 144.51 |
| 4 | 136.43 | 136.39 | 136.51 | 136.68 | 135.51 |
| 5 | 159.01 | 159.08 | 159.36 | 159.16 | 159.11 |
| 6 | 103.50 | 103.58 | 103.43 | 102.39 | 103.38 |
| 7 | 170.38 | 170.64 | 169.52 | 170.76 | 170.34 |
| 8 | 94.63 | 94.60 | 94.80 | nd | 95.08 |
| 9 | 157.97 | 157.80 | 157.77 | 157.91 | 157.71 |
| 10 | 113.78 | 113.39 | 113.09 | 113.59 | 113.36 |
| 1′ | 121.26 | 121.10 | 122.30 | 119.87 | 120.00 |
| 2′ | 118.60 | 115.19 | 108.83 | 110.83 | 112.11 |
| 3′ | 147.53 | 149.76 | 149.58 | 149.85 | 147.73 |
| 4′ | 154.93 | 156.77 | 147.43 | 146.39 | 144.89 |
| 5′ | 117.44 | 116.05 | 144.48 | 149.85 | 147.73 |
| 6′ | 128.33 | 130.04 | 115.05 | 110.83 | 112.11 |
| OMe | | 56.87 | 57.29 | 57.34 | |
| *3-O-glucoside* | | | | | |
| 1″ | 101.33 | 101.82 | 101.77 | 103.77 | |
| 2″ | 81.66 | 81.37 | 82.54 | 74.98 | |
| 3″ | 78.11 | 78.15 | 77.94 | 77.61 | |
| 4″ | 70.98 | 70.98 | 70.87 | 71.37 | |
| 5″ | 77.37 | 77.45 | 77.53 | 78.18 | |
| 6″ | 67.62 | 67.71 | 67.73 | 67.85 | |
| *2″-O-xylosyl* | | | | | |
| 1‴ | 105.75 | 105.43 | 105.20 | | |
| 2‴ | 75.75 | 75.60 | 75.68 | | |
| 3‴ | 71.63 | 71.63 | 71.74 | | |
| 4‴ | 70.99 | 70.99 | 70.92 | | |
| 5‴ | 67.23 | 67.14 | 67.01 | | |
| *6″-O-rhamnosyl* | | | | | 3-*O*-rhamnoside |
| 1′$^{v}$ | 102.17 | 102.21 | 102.30 | 102.26 | 102.64 |
| 2′$^{vv}$ | 71.90 | 70.89 | 71.93 | 71.91 | 71.55 |
| 3′$^{v}$ | 72.45 | 72.44 | 72.52 | 72.48 | 72.31 |
| 4′$^{v}$ | 73.94 | 73.98 | 73.93 | 73.90 | 73.31 |
| 5′$^{v}$ | 69.80 | 69.82 | 69.83 | 69.80 | 72.16 |
| 6′$^{v}$ | 17.87 | 17.88 | 17.89 | 17.88 | 17.95 |

[nd] Not detected.

methoxyl group and C-3′ (4.1/149.6 ppm) in the HMBC spectrum confirmed the identity of the aglycone to be petunidin. The HMBC spectrum further revealed the connectivities between the aglycone and the 3-glucosyl (H-1″/C-3, 5.6/145.3 ppm), between the 3-glucosyl and the 2″-xylosyl (H2″/C1‴, 4.0/105.2 ppm) and the 6″-rhamnosyl (H6″A/C1$^{IV}$, 4.0/102.3 ppm). Thus, **2** was found to be petunidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranoside).

Based on similar $^{1}$H and $^{13}$C NMR assignments as used for **2** and **4** (Tables 2 and 3), the identities of **1**, **3** and **5** were found to be cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl- 2″–β-xylopyranosyl-β-glucopyranoside), delphinidin 3-rhamnopyranoside and malvidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), respectively.

## 4. Conclusion

Pigments **2** and **4**, the 3-*O*-(6″-*O*-α-rhamnpyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of petunidin and peonidin, respectively, are novel compounds. Other anthocyanidin 3-*O*-(6″-rhamnosyl-2″-xylosylglucosides) seem to be relatively rare in nature. The corresponding derivative of delphinidin has been found in flowers of *Linum grandiflorum* cv Scarlet Flax (Toki, Saito, Harada, Shigihara & Honda, 1995), while the cyanidin derivative has a more widespread distribution, including the genera *Ribes* and *Rubus* (Mazza & Miniati, 1993). Also malvidin 3-*O*-(6″-*O*-α-rhamnpyranosyl-β-glucopyranosides), **3**, and delphinidin 3-rhamnopyranoside, **5**, seem to have a rather limited occurrence in plants (Harborne & Grayer, 1988; Strack & Wray, 1994).

The anthocyanidin 3-triglycosides constitute less than 2% of the total anthocyanin content, while the anthocyanidin 3-diglycosides account for 14%, where 1% is malvidin 3-rutinoside and the rest are the 3-sambubiosides (Cabrita & Andersen, in press). The monoglycosides altogether contribute with 86%, where 3.9% is delphinidin 3-rhamnoside. The pigments described in this paper account for approximately 7% of the anthocyanin content in *Vaccinium padifolium*.

From a chemotaxonomic point of view it is interesting to note the presence of anthocyanidin 3-triglycosides and 3-disaccharides together with delphinidin 3-rhamnoside in *V. padifolium*, since none of these anthocyanin classes has been identified in any other *Vaccinium* species before. The berries of these latter species seem to be characterized only by anthocyanidin 3-monoglucosides, 3-monogalactosides and 3-monoarabinosides (Mazza & Miniati, 1993). However, most *Vaccinium* species contain a complex anthocyanin mixture, which is difficult to separate in a scale necessary for proper pigment elucidation, at least for pigments occurring in relatively low amounts.

## Acknowledgements

LC is grateful to Mr Victor Freitas (Univ. Madeira) for collecting berries of *Vaccinium padifolium* and to FCT (Fundação para a Ciência e Tecnologia, Portugal) for a PRAXIS XXI BD scholarship.

## References

Cabrita, L., & Andersen, Ø. M. (1999). Anthocyanins in blue berries of *Vaccinium padifolium*. *Phytochemistry*, Accepted.

Harborne, J. B., & Grayer, R. (1988). In J. B. Harborne, *The flavonoids: advances in research since 1980* (pp. 1–20), New York: Chapman and Hall (Chapter 1).

Mazza, G., & Miniati, E. (1993). *Anthocyanins in fruits, vegetables and grains*. London: CRC Press.

Rivera, D., & Obón, C. (1995). The ethnopharmacology of Madeira and Porto Santo islands, a review. *J. Ethnopharmacology, 46*, 73–93.

Strack, D., & Wray, V. (1994). In J. B. Harborne, *The flavonoids: advances in research since 1986* (pp. 1–22). London: Chapman and Hall (Chapter 1).

Toki, K., Saito, N., Harada, K., Shigihara, A., & Honda, T. (1995). Delphinidin 3-xylosylrutinoside in petals of *Linum grandiflorum*. *Phytochemistry, 39*, 243–245.

Vieira, R. (1992). *O interesse das plantas endémicas macaronésicas* (p. 137). Serviço Nacional de Parques, Reservas e Conservação da Natureza (SNPRCN).